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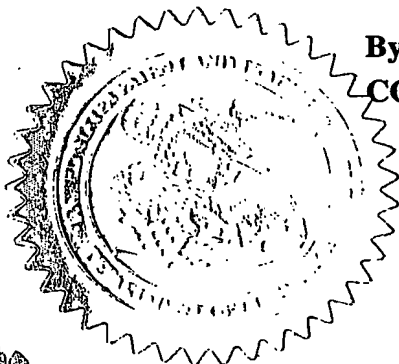
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FILING DATE UNDER 35 USC 111.**

APPLICATION NUMBER: 60/530,890**FILING DATE: December 22, 2003****PRIORITY
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PROVISIONAL APPLICATION COVER SHEET

To the Commissioner of Patents and Trademarks

Washington, DC 20231

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket No. 14567		Type a plus sign (+) inside this box -	+
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TITLE OF THE INVENTION (280 characters max)

BENZIMIDAZOLE DERIVATIVES: PREPARATION AND PHARMACEUTICAL APPLICATIONS

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ENCLOSED APPLICATION PARTS (check all that apply)

<input checked="" type="checkbox"/> Specification	45 Pages	Small Entity Statement	_____
<input checked="" type="checkbox"/> Drawing(s)	0		

METHOD OF PAYMENT (check one)

<input checked="" type="checkbox"/> A check or money order is enclosed to cover the Provisional filing fees	Provisional filing fee amount(s)	\$ 160.00
<input checked="" type="checkbox"/> Applicant(s) claim small entity status		
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees and credit any overages to Deposit Account Number: 04-1577		

Respectfully submitted,

Signature: [Signature] Date: December 22, 2003Typed or Printed Name: Ralph A. Dowell Registration No.: 26,868

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Benzimidazole derivatives: preparation and pharmaceutical applications

FIELD

Embodiments are disclosed of hydroxamate compounds which are inhibitors of histone deacetylase. More particularly, there are disclosed certain benzimidazole containing compounds and methods for their preparation. These compounds may be useful as medicaments for the treatment of proliferative diseases.

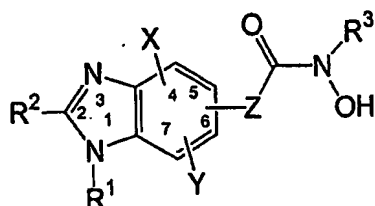
BACKGROUND

Local chromatin architecture is generally recognized as an important factor in the regulation of gene expression. The architecture of chromatin, a protein-DNA complex, is strongly influenced by post-translational modifications of the histones which are the protein components. Reversible acetylation of histones is a key component in the regulation of gene expression by altering the accessibility of transcription factors to DNA. In general, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with repression of gene expression [1,2]. In normal cells, histone deacetylases (HDACs) and histone acetyltransferase together control the level of acetylation of histones to maintain a balance. Inhibition of HDACs results in the accumulation of hyperacetylated histones, which results in a variety of cellular responses, such as apoptosis, necrosis, differentiation, inhibition of proliferation and cytostasis.

Inhibitors of HDAC have been studied for their therapeutic effects on cancer cells. For example, suberoylanilide hydroxamic acid (SAHA) is a potent inducer of differentiation and/or apoptosis in murine erythroleukemia, bladder, and myeloma cell lines [3,4]. SAHA has been shown to suppresses the growth of prostate cancer cells *in vitro* and *in vivo* [5]. Other inhibitors of HDAC that have been widely studied for their anti-cancer activities are trichostatin A (TSA) and trapoxin B [6,7]. Trichostatin A is a reversible inhibitor of mammalian HDAC. Trapoxin B is a cyclic tetrapeptide, which is an irreversible inhibitor of mammalian HDAC. However, due to the *in vivo* instability of these compounds they are less desirable as anti-cancer drugs. Recently, other small molecule HDAC inhibitors have become available for clinical evaluation [8]. Additional HDAC inhibiting compounds have been reported in the literature [Bouchain G. et al, J. Med. Chem., 46, 820-830 (2003)] and patents [WO 03/066579A2]. HDAC inhibitors have been reported to interfere with neurodegenerative processes, for instance, HDAC inhibitors arrest polyglutamine-dependent neurodegeneration [Nature, 413, 18 October, 2001].

SUMMARY

There are disclosed histone deacetylase inhibitor compounds that may be useful as pharmaceutical agents having the formula (I):



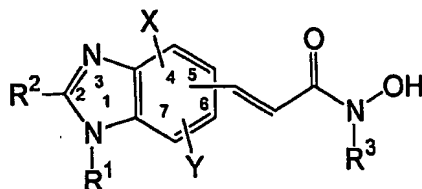
Formula I

wherein

- R¹ is selected from hydrido, C₁-C₁₀ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C₄-C₉ heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR⁴, -C(O)OH, -SH, and acyl;
- R² is H, halo, or is selected from the group consisting of C₁-C₁₀ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C₄-C₉ heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR⁴, -C(O)OH, -SH, and acyl;
- R³ is selected from H, C₁-C₆ alkyl, acyl;
- X and Y are the same or different and independently selected from hydrido, halo, C₁-C₄ alkyl, such as -CH₃ and -CF₃, -NO₂, -C(O)R⁴, -OR⁵, -SR⁵, -CN, and -NR⁶R⁷; X and Y are each attached to ring position 4, 5, 6 or 7 of Formula I;
- R⁴ is selected from C₁-C₄ alkyl;
- R⁵ is selected from C₁-C₄ alkyl, heteroalkyl, acyl;
- R⁶ and R⁷ are the same or different and independently selected from hydrido, C₁-C₆ alkyl, C₄-C₉ cycloalkyl, C₄-C₉ heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

- Z is a single bond or is selected from $-\text{CH}_2-$, $-\text{CH}_2\text{CH}_2-$, $-\text{CH}=\text{CH}-$, unsubstituted or substituted with one or more substituents independently selected from the group consisting of C_1 - C_4 alkyl; Z is attached to the ring position 4, 5, 6 or 7 of Formula I; or a pharmaceutically acceptable salt thereof.

One suitable genus of hydroxamic compounds are those of formula Ia:



Formula Ia

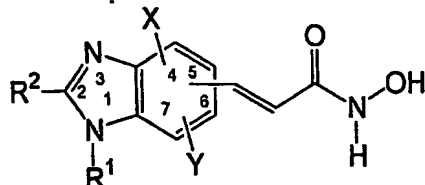
wherein

- R^1 is selected from hydrido, C_1 - C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 - C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; $=\text{O}$; $=\text{S}$; $-\text{CN}$; and $-\text{NO}_2$; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, $-\text{C}(\text{O})\text{OR}^4$, $-\text{C}(\text{O})\text{OH}$, $-\text{SH}$, and acyl;
- R^2 is H, halo, or is selected from the group consisting of C_1 - C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 - C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; $=\text{O}$; $=\text{S}$; $-\text{CN}$; and $-\text{NO}_2$; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, $-\text{C}(\text{O})\text{OR}^4$, $-\text{C}(\text{O})\text{OH}$, $-\text{SH}$, and acyl;
- R^3 is selected from H, C_1 - C_6 alkyl and acyl;
- X and Y are the same or different and independently selected from hydrido, halo, C_1 - C_4 alkyl, such as $-\text{CH}_3$ and $-\text{CF}_3$, $-\text{NO}_2$, $-\text{C}(\text{O})\text{R}^4$, $-\text{OR}^5$, $-\text{SR}^5$, $-\text{CN}$, and $-\text{NR}^6\text{R}^7$; X and Y are each attached to ring position 4, 5, 6 or 7 of Formula Ia;
- R^4 is selected from C_1 - C_4 alkyl;
- R^5 is selected from C_1 - C_4 alkyl, heteroalkyl, acyl;

- R^6 and R^7 are the same or different and independently selected from hydrido, C_1 - C_6 alkyl, C_4 - C_9 cycloalkyl, C_4 - C_9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

or a pharmaceutically acceptable salt thereof.

Another group of useful compounds are those of the formula 1b :



Formula 1b

wherein

- R^1 is selected from hydrido, C_1 - C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 - C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen, =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR⁴, -C(O)OH, -SH, and acyl;
- R^2 is H, halo, or is selected from the group consisting of C_1 - C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 - C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR⁴, -C(O)OH, -SH, and acyl;
- X and Y are the same or different and independently selected from hydrido, halo, C_1 - C_4 alkyl, such as -CH₃ and -CF₃, -NO₂, -C(O)R⁴, -OR⁵, -SR⁵, -CN, and -NR⁶ R⁷; X and Y are each attached to ring position 4, 5, 6 or 7 of Formula 1b;
- R^4 is selected from C_1 - C_4 alkyl;
- R^5 is selected from C_1 - C_4 alkyl, heteroalkyl, acyl;
- R^6 and R^7 are the same or different and independently selected from hydrido, C_1 - C_6 alkyl, C_4 - C_9 cycloalkyl, C_4 - C_9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

or a pharmaceutically acceptable salt thereof.

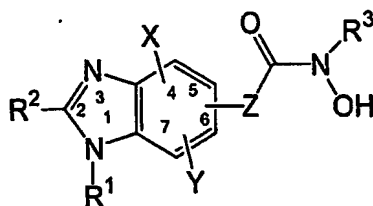
In addition to compounds of Formula I, the embodiments disclosed are also directed to pharmaceutically acceptable salts, pharmaceutically acceptable prodrugs, and pharmaceutically active metabolites of such

compounds, and pharmaceutically acceptable salts of such metabolites. Such compounds, salts, prodrugs and metabolites are at times collectively referred to herein as "HDAC inhibiting agents".

The embodiments disclosed also relate to pharmaceutical compositions each comprising a therapeutically effective amount of a HDAC inhibiting agent of the embodiments described with a pharmaceutically acceptable carrier or diluent for treating cellular proliferative ailments. The term "effective amount" as used herein indicates an amount of compound necessary to administer to a host to achieve a therapeutic result, e.g. inhibition of proliferation of malignant cancer cells, benign tumor cells or other proliferative cells.

DETAILED DESCRIPTION OF THE EMBODIMENTS

There are disclosed hydroxamate compounds, for example benzimidazoles containing hydroxamic acid in one of the substituents, that may be inhibitors of deacetylases, including but not limited to inhibitors of histone deacetylases [2]. The hydroxamate compounds may be suitable for treating tumors, including cancerous tumors. The hydroxamate compounds of the present embodiments have the following structure (I):



Formula I

wherein

- R¹ is selected from hydrido, C₁-C₁₀ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C₄-C₉ heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR⁴, -C(O)OH, -SH, and acyl;
- R² is H, halo, or is selected from the group consisting of C₁-C₁₀ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C₄-C₉ heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy,

alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, $-C(O)OR^4$, $-C(O)OH$, $-SH$, and acyl;

- R^3 is selected from H, C_1 - C_6 alkyl, acyl;
- X and Y are the same or different and independently selected from hydrido, halo, C_1 - C_4 alkyl, such as $-CH_3$ and $-CF_3$, $-NO_2$, $-C(O)R^4$, $-OR^5$, $-SR^5$, $-CN$, and $-NR^6 R^7$; X and Y are each attached to ring position 4, 5, 6 or 7 of Formula 1;
- R^4 is selected from C_1 - C_4 alkyl;
- R^5 is selected from C_1 - C_4 alkyl, heteroalkyl, acyl;
- R^6 and R^7 are the same or different and independently selected from hydrido, C_1 - C_6 alkyl, C_4 - C_9 cycloalkyl, C_4 - C_9 heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;
- Z is a single bond or is selected from $-CH_2-$, $-CH_2CH_2-$, $-CH=CH-$, unsubstituted or substituted with one or more substituents independently selected from the group consisting of C_1 - C_4 alkyl; Z is attached to ring position 4, 5, 6 or 7 of Formula 1

or a pharmaceutically acceptable salt thereof.

As used herein, the term unsubstituted means that there is no substituent or that the only substituents are hydrogen.

The term "Halogen" represents chlorine, fluorine, bromine or iodine.

The term "Halo" represents fluoro, chloro, bromo and iodo.

The term "Alkyl" refers to a straight or branched C_1 - C_{14} alkyl, including C_1 - C_6 unless otherwise noted. Examples of suitable straight and branched C_1 - C_6 alkyl substituents include methyl, ethyl, n-propyl, 2-propyl, n-butyl, sec-butyl, t-butyl, hexyl, and the like.

The term "Acyl" denotes a radical provided by the residue after removal of hydroxyl from an organic acid, examples of such radical being acetyl and benzoyl.

The term "Cycloalkyl" refers to a saturated or partially saturated, monocyclic or fused or spiro polycyclic, carbocycle from C_3 - C_9 per ring, such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and the like, unless otherwise specified.

The above discussion of alkyl and cycloalkyl substituents also applies to the alkyl portions of other substituents, such as without limitation, alkoxy, alkyl amines, alkyl ketones, arylalkyl, heteroarylalkyl, alkylsulfonyl and alkyl ester substituents and the like.

The term "Heterocycloalkyl" refers to a 3 to 9 membered aliphatic rings, such as 4 to 7 membered aliphatic rings, containing from one to three heteroatoms selected from nitrogen, sulfur, oxygen. Examples of suitable heterocycloalkyl substituents include pyrrolidyl, tetrahydrofuryl, tetrahydrothiofuranyl, piperidyl, piperazyl, tetrahydropyranyl, morpholino, 1,3-diazapane, 1,4-diazapane, 1,4-oxazepane, and 1,4-oxathiapane.

The term "Heteroalkyl" refers to a straight- or branched-chain alkyl group having from 2 to 12 atoms in the chain, one or more of which is a heteroatom selected from S, O, and N. Exemplary heteroalkyls include alkyl ethers, secondary and tertiary alkyl amines, alkyl sulfides, and the like.

The term "Aryl" refers to a monocyclic, or fused polycyclic, aromatic carbocycle (ring structure having ring atoms that are all carbon) having from 5 to 12 atoms per ring. Examples of aryl groups include phenyl, naphthyl, and the like.

The term "Heteroaryl" refers to a monocyclic, or fused polycyclic, aromatic heterocycle (ring structure having a 5 to 7 member aromatic ring containing one or more heteroatoms selected from N, O and S). Typical heteroaryl substituents include furyl, thienyl, pyrrole, pyrazole, triazole, thiazole, oxazole, pyridine, pyrimidine, isoxazolyl, pyrazine, indole, benzimidazole, and the like.

In Formula I, as well as in Formulae Ia-Ib defining sub-sets of compounds within Formula I, there is shown a benzimidazole ring system. Within this ring system, there are substitutable positions at the 4-, 5-, 6-, and 7-ring positions. In each of Formulae I, Ia, and Ib, there is a requirement for attachment of an acidic moiety at one of the ring positions. This acidic moiety may be provided by but is not limited to groups containing, a hydroxamic acid or salt derivatives of such acid which when hydrolyzed would provide the acidic moiety. In some embodiments the acidic moiety may be attached to the ring position through an alkylene group such as $-CH_2-$ or $-CH_2CH_2-$, or an alkenyl group such as $-CH=CH-$. Possible positions for attachment of the acidic moiety are the 5- and 6-ring positions.

It is understood that included in the family of compounds of Formula I are isomeric forms including diastereoisomers, enantiomers, tautomers, and geometrical isomers in "E" or "Z" configurational isomer or a mixture of E and Z isomers. It is also understood that some isomeric forms such as diastereomers, enantiomers, and geometrical isomers can be separated by physical and/or chemical methods and by those skilled in the art.

Some of the compounds of the disclosed embodiments may exist as single stereoisomers, racemates, and/or mixtures of enantiomers and/or diastereomers. All such single stereoisomers, racemates and mixtures thereof are intended to be within the scope of the subject matter described and claimed.

Additionally, Formula I is intended to cover, where applicable, solvated as well as unsolvated forms of the compounds. Thus, each formula includes compounds having the indicated structure, including the hydrated as well as the non-hydrated forms.

In addition to compounds of the Formula I, the HDAC inhibiting agents of the various embodiments include pharmaceutically acceptable salts, prodrugs, and active metabolites of such compounds, and pharmaceutically acceptable salts of such metabolites.

The term "Pharmaceutically acceptable salts" refers to salts that retain the desired biological activity of the above-identified compounds, and include pharmaceutically acceptable acid addition salts and base addition salts. Suitable pharmaceutically acceptable acid addition salts of compounds of Formula I may be prepared from an inorganic acid or from an organic acid. Examples of such inorganic acids are hydrochloric, sulfuric, and phosphoric acid. Appropriate organic acids may be selected from aliphatic, cycloaliphatic, aromatic, heterocyclic carboxylic and sulfonic classes of organic acids, examples of which are formic, acetic, propionic, succinic, glycolic, gluconic, lactic, malic, tartaric, citric, fumaric, maleic, alkyl sulfonic, arylsulfonic. Suitable pharmaceutically acceptable base addition salts of compounds of

Formula I include metallic salts made from lithium, sodium, potassium, magnesium, calcium, aluminum, and zinc, and organic salts made from organic bases such as choline, diethanolamine, morpholine. Other examples of organic salts are: ammonium salts, quaternary salts such as tetramethylammonium salt; amino acid addition salts such as salts with glycine and arginine. Additional information on pharmaceutically acceptable salts can be found in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Co., Easton, PA 1990. In the case of agents that are solids, it is understood by those skilled in the art that the inventive compounds, agents and salts may exist in different crystalline or polymorphic forms, all of which are intended to be within the scope of the present invention and specified formulae.

Possible HDAC inhibiting agents include those having an IC₅₀ value of 1 μ M or less.

Administration of compounds within Formula I to humans can be by any of the accepted modes for enteral administration such as oral or rectal, or by parenteral administration such as subcutaneous, intramuscular, intravenous and intradermal routes. The active compound is included in a pharmaceutically acceptable carrier or diluent and in an amount sufficient to deliver to the patient a therapeutically effective dose. In various embodiments the inhibitor compound may be selectively toxic or more toxic to rapidly proliferating cells, e.g. cancerous tumors, than to normal cells.

A preferred dosage will be a range from about 0.01 to 300 mg per kilogram of body weight per day. A more preferred dosage will be in the range from 0.1 to 100 mg per kilogram of body weight. A suitable dose can be administered in multiple sub-doses per day.

The active compound is usually administered in pharmaceutically-acceptable formulation. Such formulations may comprise the active compound together with one or more pharmaceutically-acceptable carriers, which may be selected from diluents and excipients that facilitate processing of the active compounds into the final pharmaceutical preparations. In certain embodiments the compositions may contain about 1 to 99% of the active ingredient. Optionally, other therapeutic agents may also be present in the formulation, including but not limited by pharmaceutical compositions comprising one or more of the above-described compounds as active ingredient.

Formulations for oral administration may be in the form of tablets and capsules containing the active compound dispersed in a binder such as gelatin, pectin, magnesium stearate, hydroxypropylmethyl cellulose and the like, together with one or more of a lubricant, preservative, surface-active or dispersing agent. Such capsules or tablets may contain controlled-release formulation as may be provided in a dispersion of active compound in hydroxypropylmethyl cellulose, glyceryl monostearate and the like. Various controlled-release materials have been established and are known by those skilled in the art.

Formulations for parenteral administration may be in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. In certain embodiments, the compounds may be formulated for parenteral administration by bolus injection or continuous infusion. Formulations for injection may be presented in unit-dosage form, for example, in ampoules or in multi-dose containers. These solutions and suspensions may be prepared from sterile powders, granules, or tablets having one or more of the carriers or diluents mentioned for use in the formulations for oral administration.

In addition to the formulations described above, the compounds may also be formulated with suitable polymeric or hydrophobic materials as a depot preparation which is long-acting and may be administered by implantation. Other delivery systems, especially for hydrophobic pharmaceutical compounds, are liposomes and emulsions which are known delivery vehicles or carriers for hydrophobic drugs.

As discussed above, the compounds of the embodiments disclosed inhibit histone deacetylases. The enzymatic activity of a histone deacetylase can be measured using known methodologies [6,10]. In certain embodiments, the histone deacetylase inhibitor interacts with and reduces the activity of more than one known histone deacetylase in the cell. In some other embodiments, the histone deacetylase inhibitor interacts and reduces the activity of predominately one histone deacetylase, for example HDAC-8, or HDAC-1. Certain preferred histone deacetylase inhibitors are those that interact with, and reduce the activity of a histone deacetylase which is involved in tumorigenesis, and these compounds may be useful for treating proliferative diseases. Examples of such cell proliferative diseases or conditions include cancer and/or any metastases, psoriasis, and restenosis. The inventive compounds may be particularly useful for treating tumors such as breast cancer, lung cancer, ovarian cancer, prostate cancer, head and/or neck cancer, or renal, gastric, and brain cancer. In addition, the inventive compounds may be useful for treating a proliferative disease that is refractory to the treatment with other chemotherapeutics; and for treating hyperproliferative condition such as leukemias, psoriasis, restenosis.

Additionally compounds of the various embodiments disclosed herein may be useful for treating neurodegenerative diseases, and inflammation.

The histone deacetylase inhibitors of the invention have significant antiproliferative effects and promotes differentiation, for example, cell cycle arrest in the G1 or G2 phase, and apoptosis

SYNTHESIS OF DEACETYLASE INHIBITORS

The agents of the various embodiments may be prepared using the reaction routes and synthesis schemes as described below, employing the techniques available in the art using starting materials that are readily available. The preparation of particular compounds of the embodiments is described in detail in the following examples, but the artisan will recognize that the chemical reactions described may be readily adapted to prepare a number of other agents of the various embodiments. For example, the synthesis of non-exemplified compounds may be successfully performed by modifications apparent to those skilled in the art, e.g. by appropriately protecting interfering groups, by changing to other suitable reagents known in the art, or by making routine modifications of reaction conditions. A list of suitable protecting groups in organic synthesis can be found in T.W. Greene's Protective Groups in Organic Synthesis, John Wiley & Sons, 1981. Alternatively, other reactions disclosed herein or known in the art will be recognized as having applicability for preparing other compounds of the various embodiments.

Reagents useful for synthesizing compounds may be obtained or prepared according to techniques known in the art.

In the examples described below, unless otherwise indicated, all temperatures in the following description are in degrees Celsius and all parts and percentages are by weight, unless indicated otherwise. Various starting materials and other reagents were purchased from commercial suppliers, such as Aldrich Chemical Company or Lancaster Synthesis Ltd., and used without further purification, unless otherwise indicated. Tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) were purchased from Aldrich in SureSeal bottles and used as received. All solvents were purified by using standard methods in the art, unless otherwise indicated.

The reactions set forth below were performed under a positive pressure of nitrogen, argon or with a drying tube, at ambient temperature (unless otherwise stated), in anhydrous solvents, and the reaction flasks are fitted with rubber septa for the introduction of substrates and reagents via syringe. Glassware was oven-dried and/or heat-dried. Analytical thin-layer chromatography was performed on glass-backed silica gel 60 F 254 plates (E Merck (0.25 mm)) and eluted with the appropriate solvent ratios (v/v). The reactions were assayed by TLC and terminated as judged by the consumption of starting material.

The TLC plates were visualized by UV absorption or with a p-anisaldehyde spray reagent or a phosphomolybdic acid reagent (Aldrich Chemical, 20wt% in ethanol) which was activated with heat, or by staining in iodine chamber. Work-ups were typically done by doubling the reaction volume with the reaction solvent or extraction solvent and then washing with the indicated aqueous solutions using 25% by volume of the extraction volume (unless otherwise indicated). Product solutions were dried over anhydrous sodium sulfate prior to filtration, and evaporation of the solvents was under reduced pressure on a rotary evaporator and noted as solvents removed in vacuo. Flash column chromatography [Still et al, J. Org. Chem., 43, 2923 (1978)] was conducted using E Merck-grade flash silica gel (47-61 mm) and a silica gel:crude material ratio of about 20:1 to 50:1, unless otherwise stated. Hydrogenolysis was done at the pressure indicated or at ambient pressure.

¹H NMR spectra was recorded on a Bruker instrument operating at 400 MHz, and ¹³C-NMR spectra was recorded operating at 100 MHz. NMR spectra are obtained as CDCl₃ solutions (reported in ppm), using chloroform as the reference standard (7.25 ppm and 77.00 ppm) or CD₃OD (3.4 and 4.8 ppm and 49.3 ppm), or an internal tetramethylsilane standard (0.00 ppm) when appropriate. Other NMR solvents were used as needed. When peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of triplets.

Coupling constants, when given, are reported in Hertz.

Mass spectra were obtained using LC/MS either in ESI or APCI. All melting points are uncorrected.

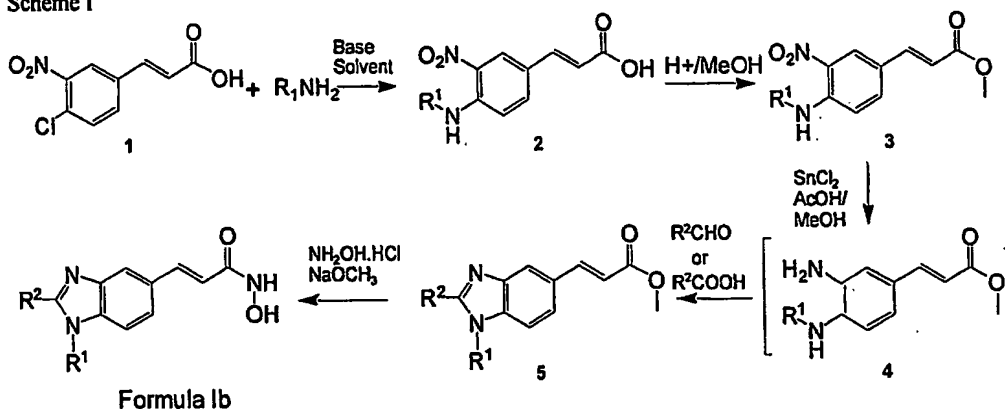
All final products had greater than 90% purity (by HPLC at wavelengths of 220 nm and 254 nm).

The following examples are intended to illustrate the embodiments disclosed and are not to be construed as being limitations thereto. Additional compounds, other than those described below, may be prepared using the following described reaction scheme or appropriate variations or modifications thereof.

SYNTHESIS

Scheme 1 illustrates the procedure used for preparing compounds of formula Ib, wherein X and Y are hydrogens. Compounds of formula I can be prepared by analogous procedure, for example, by the choice of appropriate starting material. For example, in the case of Z is -CH₂- in Formula I, such compound(s) can be synthesized by analogous method illustrated in Scheme I starting with a substituted phenylacetic acids (e.g. 3-nitro-4-chlorophenylacetic acid), appropriate amine component (R¹NH₂), aldehyde or carboxylic acid component (R²CHO or R²COOH), and appropriate hydroxylamine or N-alkyl hydroxylamine (NHR³OH where R³ is defined as above).

Scheme 1



Specifically, the hydroxamate compounds Formula Ib can be synthesized by the synthetic route shown in Scheme 1. The reaction of *trans*-4-chloro-3-nitrocinnamic acid (1) with an amine in the presence of a base (e.g. triethylamine) in an appropriate solvent (e.g. dioxane) gave (2). Treatment of (2) in methanol under acid catalysis (e.g. sulfuric acid) resulted in esterification providing (3). The nitro group of (3) can be reduced by appropriate reducing agent (e.g. tin chloride) and the resulting phenylenediamine was cyclized with an aldehyde to give (5). The hydroxamate compounds were obtained by a known synthesis method (J. Med. Chem., 2002, 45, 753-757). An alternative method for preparation of (5) is by coupling (4) with an appropriate acid and then cyclized by heating with acetic acid (J. Med. Chem. 2001, 44, 1516-1529).

The following preparation and examples are given to enable those skilled in the art to more clearly understand and to practice the subject matter hereof. They should not be considered as limiting the scope of the disclosure, but merely as being illustrative and representative thereof.

Example 1**Preparation of N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-phenyl-propyl)-1H-benzimidazol-5-yl]-acrylamide (1)****Step 1**

To a pre-stirred solution of *trans*-4-chloro-3-nitrocinnamic acid (1.0g, 4.4 mmol) in dioxane (10ml) was added triethylamine (2ml), 3-amino-1-propanol (1.5 ml). The resulting solution was heated to 85°C for 19

hours and then cooled to room temperature. The solvent was removed under vacuum. Water (100 ml) was added to the residue and the pH was adjusted to 1-1.5. The precipitate was collected and washed with cold water for 2 times and dried. The product 3-[3-nitro-4-(hydroxypropylamine)-phenyl]-acrylic acid was obtained as yellow solid (1.10g, 95%). MS(m/z): 267 (MH)⁺

Step 2

Concentrated sulfuric acid (0.5 ml) was added to the solution of *trans*-4-(3-hydroxypropylamine)-3-nitrocinnamic acid, (1.10g, 3.9 mmol) and MeOH (15ml). The resulting solution was heated to reflux for 18 hours. The reaction mixture was cooled at -10° to -15°C for 3 hours. 3-[3-nitro-4-(hydroxypropylamine)-phenyl]-acrylic acid methyl ester was collected as crystalline yellow solid (1.06g, 91%). MS(m/z): 281 (MH)⁺

Step 3

To a pre-stirred solution of methyl *trans*-4-(3-hydroxypropylamine)-3-nitrocinnamate (280 mg, 1.0 mmole) and 3-phenylbutyraldehyde (500mg, 3.4 mmole) in glacial acetic acid (5ml), Tin chloride was added (1.18g, 10.0 mmoles). The resulting solution was heated to 45°C for 17 hours and then cooled to room temperature. The solvent was removed under vacuum. Water (20 ml) and dichloromethane (20 ml) was added to the residue and stirred for 30 minutes. The organic layer was dried (MgSO₄), filtered and concentrated to an oily residue. 100 ml diethyl ether was added and stirred for 4 hours. The product 3-[1-(3-Hydroxy-propyl)-2-(2-phenyl-propyl)-1*H*-benzimidazol-5-yl]-acrylic acid methyl ester was obtained in 34.9% yield (132.0 mg). MS(m/z): 379 (MH)⁺

Step 4

Sodium methoxide (30% in methanol) (782mg, 4.1 mmole) was added to a prestirred solution of 3-[1-(3-Hydroxy-propyl)-2-(2-phenyl-propyl)-1*H*-benzimidazol-5-yl]-acrylic acid methyl ester (130mg, 0.34 mmole) and hydroxylamine hydrochloride (242 mg, 3.4 mmole) in MeOH (1.5 ml). The reaction mixture was continuously stirred for 40 minutes at room temperature and then poured into a solution of ice-water containing 1.0 ml concentrated hydrochloric acid. The mixture was extracted with dichloromethane. The organic layer was dried (MgSO₄), filtered and concentrated. The desired product was separated by reverse phase preparative HPLC. After lyophilization, 7.8 mg (6%) of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-phenyl-propyl)-1*H*-benzimidazol-5-yl]-acrylamide was obtained as powder. HPLC: 96%; t_R=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.22 min; 92%. ¹H NMR (400 MHz, DMSO-d₆, δ): 1.35 (3H, d, J=6.5Hz), 1.83 (2H, m), 3.00-4.00 (6H, m), 4.33 (2H, t, J=7.1 Hz), 6.55 (1H, d, J=15.8Hz), 7.19-7.33 (5H, m), 7.62 (1H, d, J=15.8Hz), 7.70 (1H, d, J=8.60), 7.82 (1H, d, J=8.60Hz), 7.92 (1H, s), 10.15 (1H, bs), 10.33 (1H, bs). MS(m/z): 380 [MH]⁺.

Example 2**Preparation of *N*-Hydroxy-3-[1-(3,4,5-trimethoxybenzyl)-2-(2-phenyl-ethyl)-1*H*-benzimidazol-5-yl]-acrylamide (2)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 91%; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.22 min. ¹H NMR (400 MHz, DMSO-*d*₆, δ): 3.08 (2H, t, J =7.72Hz), 3.48 (2H, t, 7.72Hz), 3.63 (3H, s), 3.67 (6H, s), 5.58 (2H, s), 6.59 (2H, s), 7.22-7.31 (7H, m), 7.63 (1H, d, J =15.78Hz), 7.71 (1H, d, J =8.76Hz), 7.83 (1H, d, J =8.76Hz), 7.98 (1H, s), 11.00 (2H, bs). MS(*m/z*): 488 [MH]⁺.

Example 3**Preparation of *N*-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-methyl-1*H*-benzimidazole-5-yl]-acrylamide (3)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 92%; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.32 min. ¹H NMR (400 MHz, DMSO-*d*₆, δ): 3.87 (3H, s), 4.01 (3H, s), 5.24 (2H, s), 6.56 (1H, d=15.80Hz), 7.32-7.50 (8H, m), 7.74(1H, d, J =8.72Hz), 7.88(1H, d, J =8.72Hz), 7.94(1H, s), 10.85(1H, bs). MS (*m/z*): 431 [MH]⁺.

Example 4**Preparation of *N*-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-(3-hydroxy-propyl)-1*H*-benzimidazole-5-yl]-acrylamide (4)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 95%; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 6.82 min. ¹H NMR (400 MHz, DMSO-*d*₆, δ): 1.96 (2H, m), 3.88(3H, s), 4.48 (2H, t, J =7.12Hz), 5.24 (2H, s), 6.56 (1H, d, J =15.76Hz), 7.32-7.50(8H, m), 7.65 (1H, d, J =15.76Hz), 7.74 (1H, d, J =8.60Hz), 7.91 (1H, d, J =8.60Hz), 7.95 (1H, s), 10.85(1H, bs). MS (*m/z*): 474 [MH]⁺.

Example 5**Preparation of *N*-Hydroxy-3-[1-(2-hydroxy-ethyl)-2-(4-methoxy-phenyl)-1*H*-benzimidazole-5-yl]-acrylamide (5)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98%; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min,

gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 4.12 min. ¹H NMR (400 MHz, DMSO-d₆, δ): 3.80(2H, t, J=5.36Hz), 3.87(3H, s), 4.39(2H, t, J=5.36Hz), 6.56(1H, d, 15.72Hz), 7.17(2H, d, J=8.88Hz), 7.61(1H, d, J=8.52Hz), 7.62(1H, d, J=15.72Hz), 7.78(1H, d, J=8.52Hz), 7.88(1H, d, J=8.88Hz), 7.90(1H, s), 10.77(1H, bs). MS (m/z): 354 [MH]⁺.

Example 6

Preparation of *N*-Hydroxy-3-[1-(2,3-hydroxy-propyl)-2-(4-methoxy-phenyl)-1*H*-benzimidazole-5-yl]-acrylamide (6)

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98%, min; t_R=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 3.39 min. NMR (400 MHz, DMSO-d₆, δ): 3.90(3H, s), 4.01(1H, m), 4.35(2H, m), 4.58(2H, dd, J=2.48 and 14.48Hz), 6.62(1H, d, J=15.84Hz), 7.27(2H, d, J=8.92Hz), 7.68(1H, d, J=15.84Hz), 8.01(4H, m), 10.13(1H, bs). MS (m/z): 383 [M]⁺.

Example 7

Preparation of *N*-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-(2,3-hydroxy-propyl)-1*H*-benzimidazole-5-yl]-acrylamide (7)

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 100%, min; t_R=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 2.06 min. NMR (400 MHz, DMSO-d₆, δ): 4.04-4.38(3H, m), 4.05(3H, s), 4.49(2H, m), 5.22(2H, s), 6.55(1H, d, J=15.72 Hz), 7.29-7.94(11H, m), 8.01(1H, s). MS (m/z): 490 [MH]⁺.

Example 8

Preparation of *N*-Hydroxy-3-[1-(2,3-hydroxy-propyl)-2-(2-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide (9)

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 93.7%, min; t_R=(LC/PDA: Phenomenex Luna C18 2.0x150mm 5μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 2.61 min. NMR (400 MHz, DMSO-d₆, δ): 3.20-3.37(4H, m), 3.90(1H, m), 4.90-4.95(2H, m), 6.54(1H, d, J=15.52 Hz), 7.98(1H, s), 8.04(1H, m), 8.27(1H, m), 9.73(1H, d, J=8.0 Hz). MS (m/z): 355 [MH]⁺.

Example 9**Preparation of *N*-Hydroxy-3-[1-(2-hydroxy-ethyl)-2-(4-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide (10)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 97.0%, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 1.14 min. NMR (400 MHz, DMSO-*d*₆, δ): 3.78 (2H, t, J =5.80 Hz), 4.43 (2H, t, J =5.80 Hz), 6.50 (1H, d, J =15.80 Hz), 7.82 (2H, d, J =8.56 Hz), 7.94 (1H, s), 8.00 (2H, d, J =5.97Hz), 8.81 (2H, d, J =5.97 Hz). MS (m/z): 325 [MH]⁺.

Example 10**Preparation of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(4-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide (11)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98.2%, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 2.61 min. NMR (400 MHz, DMSO-*d*₆, δ): 1.91 (2H, m), 3.37 (2H, t, J =5.84 Hz), 4.49 (2H, t, J =7.84 Hz), 6.54 (1H, d, J =15.52 Hz), 7.98 (1H, s), 8.06 (2H, d, J =6.26 Hz), 8.90 (2H, d, J =6.26 Hz). MS (m/z): 339 [MH]⁺.

Example 11**Preparation of *N*-Hydroxy-3-[1-(3-pyridylmethyl)-2-(2-phenyl-ethyl)-1*H*-benzimidazol-5-yl]-acrylamide (12)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 97.9%, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 3.32 min. NMR (400 MHz, DMSO-*d*₆, δ): 3.11 (2H, t, J =8.40 Hz), 5.71 (2H, s), 6.51 (1H, d, J =15.80Hz), 7.20-7.31 (6H, m), 7.43 (1H, m), 7.40-7.57 (4H, m), 7.94 (1H, s), 8.57 (1H, s). MS (m/z): 399 [MH]⁺.

Example 12**Preparation of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-pyridyl)-1*H*-benzimidazol-5-yl]-acrylamide (13)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98.3%, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 3.37 min. NMR (400 MHz, DMSO-*d*₆, δ): 1.98 (2H, m), 3.30 (2H, m), 4.86 (2H, t, J =7.00 Hz), 6.51 (1H, d, J =15.76 Hz), 7.77 (2H, d, J =8.56 Hz), 7.94 (1H, s), 8.05 (1H, m), 8.30 (1H, d, J =7.92 Hz), 8.78 (1H, d, J =4.28 Hz). MS (m/z): 339 [MH]⁺.

Example 13**Preparation of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-phenethyl-1*H*-benzimidazol-5-yl]-acrylamide (14)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 97.3%, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 2.63 min. NMR (400 MHz, DMSO-*d*₆, δ): 1.87 (2H, m), 3.18 (2, t, J =7.40 Hz), 4.41 (2H, t, J =7.0 Hz), 6.57 (1H, d, J =17.60 Hz), 7.15 (5H, m), 7.64 (1, d, J =17.60 Hz), 7.89 (1H, d, J =8.64 Hz), 7.95 (1H, s). MS (m/z): 366 [MH]⁺.

Example 14**Preparation of *N*-Hydroxy-3-(2-phenethyl-1-(pyridin-2-yl)methyl-1*H*-benzimidazol-5-yl)-acrylamide (16)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 99.7%, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 3.11 min. NMR (400 MHz, DMSO-*d*₆, δ): 3.31 (2H, t, J =7.56 Hz), 5.81 (2H, s), 6.57 (1H, d, J =17.60 Hz), 7.20-7.36 (6H, m), 7.52 (1H, m), 7.64 (1H, d, J =17.60 Hz), 7.68 (1H, d, J =8.48 Hz), 7.77 (1H, d, J =8.48 Hz), 7.87 (1H, m), 8.44 (1H, d, J =3.92Hz). MS (m/z): 399 [MH]⁺.

Example 15**Preparation of *N*-Hydroxy-3-[1-(3-Dimethylamino-2,2-dimethyl-propyl)-2-phenethyl-1*H*-benzimidazol-5-yl]-acrylamide (17)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC:100% , min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 2.13 min. NMR (400 MHz, DMSO-*d*₆, δ): 1.08 (6H, s), 2.89 (6H, s), 4.30 (2H, s), 6.54 (1H, d, J =15.80), 7.03 (1H, s), 7.16 (1H, s), 7.22-7.32 (6H, m), 7.65 (1H, d, J =15.80 Hz), 7.91 (1H, s). MS (m/z): 421 [MH]⁺.

Example 16**Preparation of *N*-Hydroxy-3-[2-Benzoyloxymethyl-1-(3-hydroxy-propyl-1*H*-benzimidazol-5-yl)-acrylamide (19)**

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98.6 %, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 4.50 min. ¹H NMR (400 MHz, DMSO, δ): 1.94 (2H, m), 3.43 (2H, t, J = 5.8 Hz), 4.42 (2H, t, J = 7.2 Hz), 4.67 (2H, s), 4.97 (2H, s), 6.53 (1H, d, J = 15.8 Hz),

7.38 (5H, m), 7.63 (1H, d, $J = 15.8$ Hz), 7.67 (1H, d, $J = 9.1$ Hz), 7.80 (1H, d, $J = 8.6$ Hz), 7.90 (1H, s), 10.77 (1H, bs). MS (m/z): 382 [MH]⁺.

Example 17

Preparation of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-thiophen-3-yl-1*H*-benzimidazol-5-yl]-acrylamide (20)

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 97.9 %, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 3.06 min. ¹H NMR (400 MHz, DMSO, δ): 1.98 (2H, m), 3.49 (2H, t, $J = 5.8$ Hz), 4.56 (2H, t, $J = 7.2$ Hz), 6.56 (1H, d, $J = 15.8$ Hz), 7.65 (1H, d, $J = 15.8$ Hz), 7.69 (1H, d, $J = 8.7$ Hz), 7.75 (1H, dd, $J = 5.1$ Hz, 1.2 Hz), 7.89 (2H, m), 7.93 (1H, s), 8.42 (1H, dd, $J = 2.6$ Hz), 10.90 (1H, bs);. MS (m/z): 344 [MH]⁺.

Example 18

Preparation of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-isobutyl-1*H*-benzimidazol-5-yl]-acrylamide (21)

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 100 %, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 3.14 min. ¹H NMR (400 MHz, DMSO, δ): 1.01 (6H, d, $J = 6.6$ Hz), 1.94 (2H, m), 2.28 (1H, m), 3.04 (2H, d, $J = 7.4$ Hz), 3.47 (2H, t, $J = 5.8$ Hz), 4.46 (2H, t, $J = 7.1$ Hz), 6.56 (1H, d, $J = 15.8$ Hz), 7.65 (1H, d, $J = 15.8$ Hz), 7.73 (1H, d, $J = 8.6$ Hz), 7.89 (1H, d, $J = 8.6$ Hz), 7.94 (1H, s). MS (m/z): 314 [MH]⁺.

Example 19

Preparation of *N*-Hydroxy-3-[1-(3-hydroxy-propyl)-2-octyl-1*H*-benzimidazol-5-yl]-acrylamide (23)

Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 99.0 %, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.38 min. ¹H NMR (400 MHz, DMSO, δ): 0.86 (3H, t, $J = 6.8$ Hz), 1.32 (10H, m), 1.83 (2H, m), 1.94 (2H, m), 3.12 (2H, t, $J = 7.7$ Hz), 3.46 (2H, t, $J = 5.8$ Hz), 4.44 (2H, t, $J = 7.0$ Hz), 6.56 (1H, d, $J = 15.8$ Hz), 7.64 (1H, d, $J = 15.8$ Hz), 7.71 (1H, d, $J = 8.6$ Hz), 7.87 (1H, d, $J = 8.6$ Hz), 7.92 (1H, s). MS (m/z): 374 [MH]⁺.

Example 20

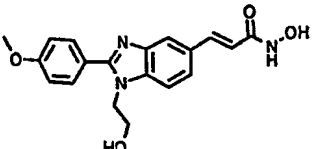
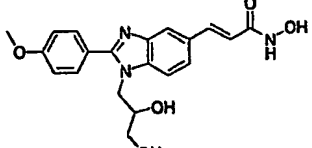
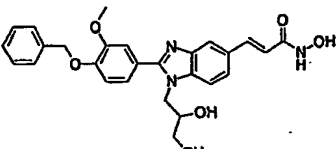
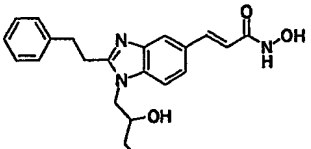
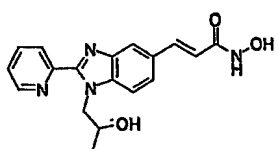
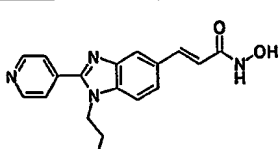
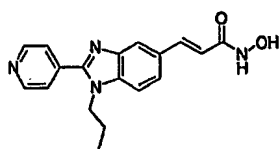
Preparation of *N*-Hydroxy-[2-cyclohexyl-1-(3-hydroxy-propyl)-1*H*-benzimidazol-5-yl]-acrylamide (24)

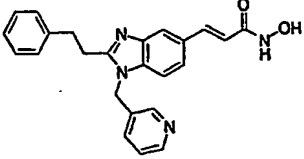
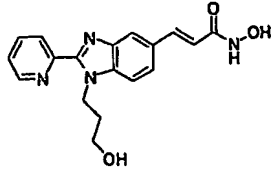
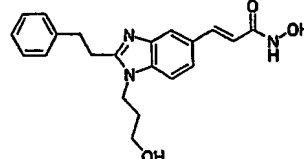
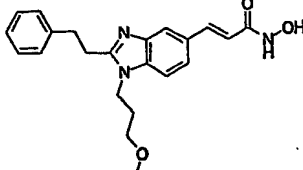
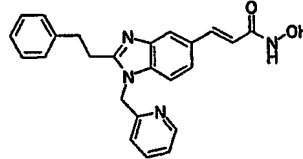
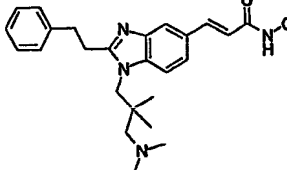
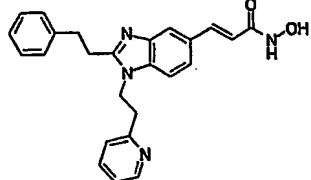
Proceeding as described in Example 1 above but using appropriate starting materials, the titled compounds was prepared. HPLC: 98.0 %, min; t_R =(LC/PDA: Phenomenex Luna C18 2.0x150mm 5 μ column; 0.8 ml/min, gradient 5-65% B over 15.5 min, Solvent A: H₂O with 0.1% trifluoroacetic acid; Solvent B: Acetonitrile with 0.1% trifluoroacetic acid; UV 254): 7.38 min. ¹H NMR (400 MHz, DMSO, δ): 1.28-2.03 (12H, m), 3.33 (1H, m), 3.47 (2H, t, J = 5.7 Hz), 4.51 (2H, t, J = 6.9 Hz), 6.58 (1H, d, J = 15.8 Hz), 7.65 (1H, d, J = 15.8 Hz), 7.76 (1H, d, J = 8.6 Hz), 7.92 (1H, d, J = 8.7 Hz), 7.93 (1H, s), 10.85 (1H, bs). MS (m/z): 344 [MH]⁺.

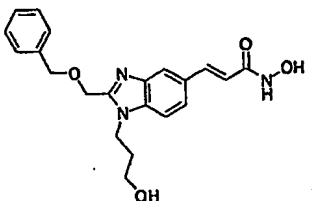
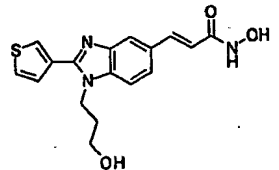
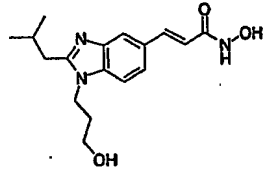
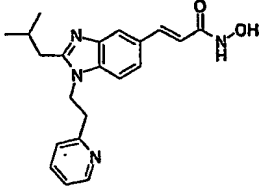
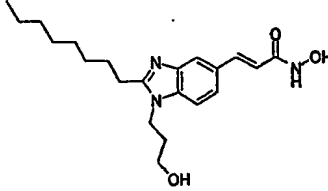
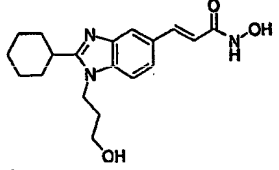
The following compounds are some representative examples prepared by methods analogous to those disclosed in above Examples :

Table 1

Compound	Structures	m/z [MH] ⁺	¹ H-NMR (400 MHz, DMSO- d_6 , δ)
1		380	1.35 (3H, d, J =6.5Hz), 1.83 (2H, m), 3.00-4.00 (6H, m), 4.33 (2H, t, J =7.1 Hz), 6.55 (1H, d, J =15.8Hz), 7.19-7.33 (5H, m), 7.62 (1H, d, J =15.8Hz), 7.70 (1H, d, J =8.60), 7.82 (1H, d, J =8.60Hz), 7.92 (1H, s), 10.15 (1H, bs), 10.33 (1H, bs).
2		488	3.08 (2H, t, J =7.72Hz), 3.48 (2H, t, 7.72Hz), 3.63 (3H, s), 3.67 (6H, s), 5.58 (2H, s), 6.59 (2H, s), 7.22-7.31 (7H, m), 7.63 (1H, d, J =15.78Hz), 7.71 (1H, d, J =8.76Hz), 7.83 (1H, d, J =8.76Hz), 7.98 (1H, s), 11.00 (2H, bs).
3		431	3.87 (3H, s), 4.01 (3H, s), 5.24 (2H, s), 6.56 (1H, d=15.80Hz), 7.32-7.50 (8H, m), 7.74(1H, d, J =8.72Hz), 7.88(1H, d, J =8.72Hz), 7.94(1H, s), 10.85(1H, bs).
4		474	1.96 (2H, m), 3.88(3H, s), 4.48 (2H, t, J =7.12Hz), 5.24 (2H, s), 6.56 (1H, d, J =15.76Hz), 7.32-7.50(8H, m), 7.65 (1H, d, J =15.76Hz), 7.74 (1H, d, J =8.60Hz), 7.91 (1H, d, J =8.60Hz), 7.95 (1H, s), 10.85(1H, bs).

5		354	3.80(2H, t, J=5.36Hz), 3.87 (3H, s), 4.39(2H, t, J=5.36Hz), 6.56(1H, d, 15.72Hz), 7.17 (2H, d, J=8.88Hz), 7.61(1H, d, J=8.52Hz), 7.62(1H, d, J=15.72Hz), 7.78(1H, d, J=8.52Hz), 7.88(1H, d, J=8.88Hz), 7.90(1H, s), 10.77(1H, bs).
6		383	3.90 (3H, s), 4.01(1H, m), 4.35(2H, m), 4.58(2H, dd, J=2.48 and 14.48Hz), 6.62(1H, d, J=15.84Hz), 7.27(2H, d, J=8.92Hz), 7.68(1H, d, J=15.84Hz), 8.01(4H, m), 10.13 (1H, bs).
7		490	4.04-4.38 (3H, m), 4.05 (3H, s), 4.49 (2H, m), 5.22 (2H, s), 6.55 (1H, d, J=15.72 Hz), 7.29-7.94 (11H, m), 8.01 (1H, s).
8		382	
9		355	3.20-3.37 (4H, m), 3.90 (1H, m), 4.90-4.95 (2H, m), 6.54 (1H, d, J=15.52 Hz), 7.98 (1H, s), 8.04 (1H, m), 8.27 (1H, m), 9.73 (1H, d, J=8.0 Hz).
10		325	3.78 (2H, t, J=5.80 Hz), 4.43 (2H, t, J=5.80 Hz), 6.50 (1H, d, J=15.80 Hz), 7.82 (2H, d, J=8.56 Hz), 7.94 (1H, s), 8.00 (2H, d, J=5.97Hz), 8.81 (2H, d, J=5.97 Hz).
11		339	1.91 (2H, m), 3.37 (2H, t, J=5.84 Hz), 4.49 (2H, t, J=7.84 Hz), 6.54 (1H, d, J=15.52 Hz), 7.98 (1H, s), 8.06 (2H, d, J=6.26 Hz), 8.90 (2H, d, J=626 Hz).

12		399	3.11 (2H, t, J=8.40 Hz), 5.71 (2H, s), 6.51 (1H, d, J=15.80Hz), 7.20-7.31 (6H, m), 7.43 (1H, m), 7.40-7.57 (4H, m), 7.94 (1H, s), 8.57 (1H, s).
13		339	1.98 (2H, m), 3.30 (2H, m), 4.86 (2H, t, J=7.00 Hz), 6.51 (1H, d, J=15.76 Hz), 7.77 (2H, d, J=8.56 Hz), 7.94 (1H, s), 8.05 (1H, m), 8.30 (1H, d, J=7.92 Hz), 8.78 (1H, d, J=4.28 Hz). MS (m/z): 339 [MH] ⁺ .
14		366	1.87 (2H, m), 3.18 (2, t, J=7.40 Hz), 4.41 (2H, t, J=7.0 Hz), 6.57 (1H, d, J=17.60 Hz), 7.15 (5H, m), 7.64 (1, d, J=17.60 Hz), 7.89 (1H, d, J=8.64 Hz), 7.95 (1H, s).
15		380	
16		399	3.31 (2H, t, J=7.56 Hz), 5.81 (2H, s), 6.57 (1H, d, J=17.60 Hz), 7.20-7.36 (6H, m), 7.52 (1H, m), 7.64 (1H, d, J=17.60 Hz), 7.68 (1H, d, J=8.48 Hz), 7.77 (1H, d, J=8.48 Hz), 7.87 (1H, m), 8.44 (1H, d, J=3.92Hz).
17		421	1.08 (6H, s), 2.89 (6H, s), 4.30 (2H, s), 6.54 (1H, d, J=15.80), 7.03 (1H, s), 7.16 (1H, s), 7.22-7.32 (6H, m), 7.65 (1H, d, J=15.80 Hz), 7.91 (1H, s).
18		413	

19		382	1.94 (2H, m), 3.43 (2H, t, $J = 5.8$ Hz), 4.42 (2H, t, $J = 7.2$ Hz), 4.67 (2H, s), 4.97 (2H, s), 6.53 (1H, d, $J = 15.8$ Hz), 7.38 (5H, m), 7.63 (1H, d, $J = 15.8$ Hz), 7.67 (1H, d, $J = 9.1$ Hz), 7.80 (1H, d, $J = 8.6$ Hz), 7.90 (1H, s), 10.77 (1H, bs).
20		344	1.98 (2H, m), 3.49 (2H, t, $J = 5.8$ Hz), 4.56 (2H, t, $J = 7.2$ Hz), 6.56 (1H, d, $J = 15.8$ Hz), 7.65 (1H, d, $J = 15.8$ Hz), 7.69 (1H, d, $J = 8.7$ Hz), 7.75 (1H, dd, $J = 5.1$ Hz, 1.2 Hz), 7.89 (2H, m), 7.93 (1H, s), 8.42 (1H, dd, $J = 2.6$ Hz), 10.90 (1H, bs).
21		318	1.01 (6H, d, $J = 6.6$ Hz), 1.94 (2H, m), 2.28 (1H, m), 3.04 (2H, d, $J = 7.4$ Hz), 3.47 (2H, t, $J = 5.8$ Hz), 4.46 (2H, t, $J = 7.1$ Hz), 6.56 (1H, d, $J = 15.8$ Hz), 7.65 (1H, d, $J = 15.8$ Hz), 7.73 (1H, d, $J = 8.6$ Hz), 7.89 (1H, d, $J = 8.6$ Hz), 7.94 (1H, s).
22		365	
23		374	0.86 (3H, t, $J = 6.8$ Hz), 1.32 (10H, m), 1.83 (2H, m), 1.94 (2H, m), 3.12 (2H, t, $J = 7.7$ Hz), 3.46 (2H, t, $J = 5.8$ Hz), 4.44 (2H, t, $J = 7.0$ Hz), 6.56 (1H, d, $J = 15.8$ Hz), 7.64 (1H, d, $J = 15.8$ Hz), 7.71 (1H, d, $J = 8.6$ Hz), 7.87 (1H, d, $J = 8.6$ Hz), 7.92 (1H, s).
24		344	1.28-2.03 (12H, m), 3.33 (1H, m), 3.47 (2H, t, $J = 5.7$ Hz), 4.51 (2H, t, $J = 6.9$ Hz), 6.58 (1H, d, $J = 15.8$ Hz), 7.65 (1H, d, $J = 15.8$ Hz), 7.76 (1H, d, $J = 8.6$ Hz), 7.92 (1H, d, $J = 8.7$ Hz), 7.93 (1H, s), 10.85 (1H, bs).

BIOLOGICAL TESTING AND ENZYME ASSAYS

Recombinant GST-HDAC1 Protein expression and purification

Human cDNA library was prepared using cultured SW620 cells. Human HDAC1 coding region was amplified from this cDNA library, and cloned into pDEST20 vector using GATEWAY Cloning Technology (Invitrogen). The pDEST20-HDAC1 construct was confirmed by DNA sequencing. pDEST20-HDAC1 was then transformed into *Escherichia coli* DH10Bac competent cells. pDEST20-HDAC1 bacmid was isolated from the positive white colony. Recombinant baculovirus was prepared using the Bac-To-Bac method following the manufacturer's instruction (Invitrogen). The steps involved transfecting SF9 cells with the isolated bacmid using CELLFECTIN reagent (Invitrogen), followed by two-round of virus amplifications. Baculovirus titer was determined by plaque assay to be about 10^8 PFU/ml.

Expression of GST-HDAC1 was done by infecting SF9 cells with pDEST20-HDAC1 baculovirus at MOI=1 for 60 h. Cells were harvested by centrifugation at 500g for 10 min at 4°C. The cell pellets were suspended in lysis buffer (4ml/g of pellet) containing 50mM HEPES, pH7.5, 250mM NaCl, 1% Triton X-100, 3mM MgCl₂, 1mM DTT, 1mM PMSF, 20µg/ml DNaseI and 1x Protease Inhibitor cocktail (Roche). Cell lysate was then subjected to 3 freeze/thaw cycles in liquid N₂/37°C water bath. Cell debris was removed from the soluble cell lysate by centrifugation at 13,200rpm for 30 min at 4°C. Soluble cell lysate was then incubated with pre-equilibrated Glutathione Sepharose 4B beads (Amersham) at 4°C for 2 h. Unbound protein was removed from the mixture by a centrifugation at 500g for 5 min at 4°C. The beads were washed with PBS buffer for 3 times. And GST-HDAC1 protein was eluted by elution buffer containing 50mM Tris, pH8.0, 150mM NaCl, 1% Triton X-100 and 10mM or 20mM reduced Glutathione. Purified GST-HDAC1 protein was dialyzed with HDAC storage buffer containing 10mM Tris, pH7.5, 100mM NaCl and 3mM MgCl₂. 20% Glycerol was added to purified GST-HDAC1 protein before storage at -80°C.

Equipments:

- (a). Baculovirus culture incubator and shaking incubator, set at 27.5°C (Sanyo, Labnet, IKA and Infors, Swiss)
- (b). Biological Safety cabinet (Gelman and NuAire)

Materials:

- (a). SF9 cells (Invitrogen)
- (b). SF-900 II SFM medium (Invitrogen)
- (c). Disposable polycarbonate Erlenmeyer flasks (Corning)
- (d). CELLFECTIN reagent (Invitrogen)
- (e). 6-well tissue culture dishes (Falcon)
- (f). Concert High Purity Plasmid Miniprep (Marligen)
- (g). Glutathione Sepharose 4B beads (Amersham)

In vitro HDAC assay for determination of IC50 valuesExperimental procedure

The assay has been carried out in 96well format and the BIOMOL fluorescent-based HDAC activity assay has been applied. Compared to the traditional HDAC assay-using radioisotope labeled substrate [9, 10, 11], this assay is more specific (p53 peptide substrate for HDAC8 [12, 13]), easier (two steps), homogenous and sensitive (fluorescent-based). Briefly, deacetylation of the substrate sensitizes it to the developer, which then generates a fluorophore (symbol). The fluorophore is excited with 360 nm light and the emitted light (460 nm) is detected on a fluorometric plate reader. Currently, this assay has been successfully applied in many studies related to HDAC inhibition effects [14, 15, 16, 17]. The analytical software, Prism 3.0 has been used to generate IC50 from a series of data. The pipetting scheme for a representative experiment is shown below: 1. add 10 ul of assay buffer into columns 2-5, 7-10, wells B,C,D,E,G11-12 ; 2. add 12.5 ul of 2.5X compound (2.5 uM) into columns 1, 6 and wells A11-12. 12.5 ul buffer into wells F11 and12; 3. serially dilute 2.5 ul (5X) in orientation as indicated above; 4. discard last 2.5 ul from column 5, 10 and wells E11-12; 5. add 2.5 ul of HDAC enzyme (0.5 U for HDAC8, 2 ul for HDAC1 to reach final concentration of 600 nM) into all wells except F11-12; 6. add 12.5 ul of 2X substrate (200 uM for peptide substrate, 500 uM for generic substrate) into all wells; 7. incubate at RT for 2 hr with agitation; 8. add 25 ul of 2X developer into all wells and incubate for 10 mins.

Equipment & MaterialsEquipment:

- (a). Tecan Ultra Microplate detection system (Tecan Group Ltd. Switzerland)
- (b). Labnet Shaker, Model 30 (National Labnet Co., Inc. Woodbridge, NJ, USA)

Materials:

- (a). 96-well U-form black microplate, 650209, (Greiner Bio-One, Frickenhausen, Germany)
- (b). Histone Deacetylase 8 (HDAC8) (human, recombinant), 100 U (SE-145, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)
- (c). *Fluor de Lys*-HDAC8 Substrate, 0.5 umol (KI-178, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)
- (d). *Fluor de Lys*TM Developer Concentrate (20x), 300 ul (KI-105, BIOMOL Research Laboratories, Inc. Plymouth Meeting, PA, USA)
- (e). HDAC8 assay buffer: Tris pH7.5, 25 mM; NaCl, 137 mM; KCl, 2.7 mM; MgCl₂, 1 mM, BSA,

Data analysis

The assay is composed of duplicates for each compound. Thus, for the raw values from fluorescence reading, a mean value will be calculated using Excel formula [average (value1: value2)]; in addition, standard deviation will also be determined based on duplicates by formula [std (value1: value2)]. The Z' factor is calculated on the basis of high/ low signal with definitions: high signal= no inhibitor and low signal= no enzyme. Hence $Z' = 1 - 3 * (SD_{high} + SD_{low}) / (Signal_{high} - Signal_{low})$.

The HDAC enzyme inhibition results of representative compounds are shown in Table 2.

Table 2

Compound	HDAC8 Enzyme Activity, IC ₅₀ (μM)	HDAC1 Enzyme Activity IC ₅₀ (uM)
1	0.119	0.051
2	0.355	0.026
3	1.71	1.37
4	0.790	1.34
5	0.401	4.32
6	0.262	1.38
7	0.336	1.52
8	0.454	0.286
9	0.344	1.34
10	0.883	2.66
11	0.161	0.846
12	0.202	0.131
13	0.141	0.385
14	0.251	0.171
15	0.313	0.206
16	0.366	0.194
17	0.353	0.388
19	0.145	0.165
20	0.537	1.91
21	0.238	0.064
23	0.402	0.529
24	0.203	3.24
SAHA	0.234	0.106

Cell-based proliferation assay for determination of GI₅₀ values

The Cell proliferation assay is performed in a 96-well plate format. Cells are plated overnight and treated with compounds (in triplicates, 9-dose treatment, 4-fold dilutions from 100uM) over 96hrs. Cell growth is then determined by analysing the number of viable cells remaining following treatment of the cells. Dose response curves are plotted to determine GI₅₀ values for the compounds. Staurosporine

treatment is used as a positive control for the experiments as staurosporine inhibits kinases and has anti-proliferative activity.

The CyQUANT cell proliferation assay is a fluorescent assay based on the measurement of cellular nucleic acid content. It contains a fluorescent nucleic acid stain, the CyQUANT GR reagent, that measures total nucleic acids as a direct indication of cell number. The assay is more rapid and convenient than conventional assays measuring metabolic activity as it does not require long incubations and cells can be frozen and stored prior to assaying. In this protocol, it is applied solely to the analysis of adherent cell lines. After treatment with compounds, detached dead cells are removed with the culture supernatant and only the viable cells remaining are quantified in the assay by fluorescence measurement at 485/535nm. In the analysis of viable cells in suspension cell lines, the CyQuant assay cannot distinguish between live and dead cells in suspension as it measures total nucleic acid content (from both live and dead cells) in solution. Thus, for analysis of suspension cell lines, methods based on measurement of metabolic activity have to be used.

The method adapted for this protocol is the Celltiter96 Aqueous One Solution Cell Proliferation Assay. It is a colorimetric method for determination of cell viability based on the cleavage of an MTS tetrazolium compound into a coloured formazan product by metabolically active cells. The quantity of formazan product is directly proportional to the number of living cells in culture. The assay has to be performed immediately after compound treatment of cells. The Celltiter96 Aqueous One Solution Reagent is added directly to the cells in culture and incubated for 1-4 hours for colour development before recording the absorbance at 490nm.

Equipment & Materials

Equipment:

Vortex Mixer

Plate shaker for 96-well plates

Single and Multichannel pipettors

Cell culture Incubator

Microplate readers for reading of fluorescence(485/535nm) and absorbance (490nm)

Materials:

Appropriate cancer cell line(s) and culture media

10mM Compounds

1mM Staurosporine

96-well cell culture plates

Reagent reservoirs

For adherent cell lines: CyQUANT cell proliferation assay kit (Molecular Probes #C-7026)

For suspension cell lines: Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega #G3580)

The cell activity results of representative compounds are shown in Table 3.

Table 3

Compound	NCI H552 (μM)	Colon 205 (μM)
1	1.31	0.52
2	1.20	0.43
4	66.51	29.87
5	>100	>100
6	>100	>100
7	>100	>100
8	>100	41.36
9	>100	>100
11	>100	>100
12	N.A.	0.38
13	N.A.	12.32
14	N.A.	3.07
15	N.A.	1.99
16	N. A.	0.94
SAHA	1.29	2.57

Histone H3 acetylation assay

A hallmark of histone deacetylase (HDAC) inhibition is the increase in the acetylation level of histones. The degree of histone acetylation can be monitored by a Western Blot approach, where specific antibodies directed against the acetylated version of histone H3 are used. Briefly, 1.5×10^6 Colo 205 colon cancer cells were plated into 10 cm dishes and grown overnight in RPMI medium. Thereafter, the cells were treated with increasing amounts of HDAC inhibitory compounds by adding them into the medium (0.1, 1, 5 and 10 μM final concentration). After 12 hours of incubation the cells were harvested, lysates prepared and the Western Blot procedure carried out as described in detail below.

Western Blot approach

Specific proteins can readily be identified with the use of antibodies directed explicitly towards it. Accordingly, the more abundant protein would display a stronger signal compared to one which was present in a lower concentration.

Proteins must first be extracted from cells and quantified before equal amounts from each cell line can be separated by gel electrophoresis (SDS-PAGE).

Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for Western Blot analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane (nitrocellulose or PVDF) in the same sequence of separation as that on the SDS-PAGE. The membrane is then blocked with an inert protein like bovine serum albumin (BSA) or non-fat milk. This will avoid non-specific binding of the primary antibody to the un-blotted surface of the membrane.

To detect the antigen (separated protein of interest) blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane.

In order to detect the antibody which has bound, an anti-immunoglobulin antibody coupled to a reporter group such as the enzyme horse radish peroxidase is added (e.g. Goat anti-human IgG- alkaline phosphatase). This anti-Ig-enzyme is commonly called a "second antibody" or "conjugate". Finally after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody is bound to the protein.

PROTEIN EXTRACTION

Keep equipment cold and everything else on ice.

PROTEIN EXTRACTION BUFFER (1 ml.):

Take 200ul each of 5x Buffer, 5x NaCl, and 5x Igepal from the Sigma Mammalian Cell Lysis Kit, and make up to volume with 400ul of deionised water. Add 60 ul Protease Inhibitor Cocktail for every ml. of extraction buffer. (*Reduce Protease Inhibitor if higher volumes are used in protein extraction ~ 15ul for every ml)

Protein Extraction from cells grown in culture:

Rinse cells in ice-cold PBS and detach with trypsin. Pellet cells by centrifugation ~1300g x 5 minutes, and remove supernatant. Resuspend cell pellet in 1 ml. PBS and transfer into a 1.5ml. Eppendorf tube and centrifuge ~ 13000g for 10 mins. Remove supernatant and gently resuspend cells in appropriate amounts of PROTEIN EXTRACTION BUFFER (e.g ~ 50ul for small pellets and 100 – 150ul for larger pellets) Immediately freeze in LN₂ and allow to thaw on ice ~ 20 mins. Centrifuge at ~13000 x g for 30 minutes Remove supernatant into a new tube and either freeze in LN₂ and store at -80° C or keep on ice for further work.

PROTEIN QUANTIFICATION (BRADFORD METHOD)

1. Prepare protein standards of 0 – 3000 ng from a stock solution of 250ug/ml; 2. dilute all samples between 50x – 100x; 3. dilute Bio-Rad Protein Assay solution 5x and transfer 200ul into each well in a 96-well plate; 4. add 20ul of each standard or sample solution into individual wells; 5. mix for ~ 1 min.; 6. read absorbance at 595 nm.

PROTEIN SEPARATION (SDS-PAGE)6X Sample Loading Buffer

7 ml. 0.5M Tris-HCl, 0.4% SDS, 3 ml. glycerol, 1 g SDS, 0.93 g DTT, 1.2 mg bromophenol blue. Make up volume to 10 ml with deionised water. Store in small aliquots at -20°C. Add an appropriate amount of sample loading buffer to all samples. Heat to 95°C in the heating block for 5 minutes.

GEL ELECTROPHORESIS:

Set up the Xcell Sure Lock Electrophoresis gel tank according to the manufacturer's instructions. Place the pre-cast NuPage gels into the gel holders and add diluted NuPage tank buffer into the chambers.

Load the Rainbow molecular weight marker and samples into respective wells in the gel

Connect the gel tank to the power pac and run the at 150V constant for ~ 1 hour.

PROTEIN TRANSFER – ELECTROBLOTTING

While gel is running cut PVDF membrane and filter papers to exact size of gel (8.5 x 6cm). Wet PVDF with methanol in petri-dish. Then soak PVDF in diluted NuPage transfer buffer for at least 15min. Take gel, disassemble and cut wells and stacking gel off. Make a gel sandwich (From black side down on bench):

- Sponge (make sure no bubbles)
- 3x Whatman filter paper (Wet in transfer buffer)
- Gel, roll out bubbles
- PVDF, roll out bubbles
- 3x Whatman filter paper (Wet in transfer buffer)
- Sponge (make sure no bubbles)

Assemble transfer apparatus. Place a small stirrer in chamber. Load transfer cassettes with the BLACK side FACING BLACK part of blotting chamber. Fill chamber with Transfer Buffer past all holes of sandwich pack. Run at 100V for 60mins or 250mA for 150 mins at 4°C. Disassemble gel sandwich.

Mark the marker on the blot according to the color and identify the protein side up.

IMMUNODETECTION (ANTIBODIES)Reagents:

10X Tris Saline Solution (TSS) (pH 7.6)

Tris 100 mM 12.1g/L

NaCl 1.5M 87.6g/L

Adjust pH to 7.6 and store at 0-4°C

Blocking Solution (1x TSS/5% milk)

10x TSS 10ml

Non-fat powder milk 5g

Deionised water 90ml

Washing Buffer (1x TSS/0.5% milk /0.1% Tween-20)

10X TSS	100ml
Non-fat powder milk	2.5g
Tween-20	1ml
Deionised water	890ml

Blocking

Transferred proteins can be visualized by staining the membrane for a few minutes with Ponceau S. Remove stain from the membrane by washing with deionised water. Place membrane into blocking solution. Block for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C.

Incubation with primary antibodyAntibodies used:

- I Anti-acetylated Histone H3 (Lysin 14), UPSTATE
- II Anti-Actin (SIGMA)

Protocol

Decant the blocking buffer and wash the membrane with washing buffer for another 30mins. Add the primary antibody, diluted in washing buffer as suggested in the product description sheet. Incubate with agitation for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C. Incubation with secondary antibody. Decant the primary antibody. Wash the membrane 3X with washing buffer. First, for 15 mins then the subsequent 2X for 5 minutes. Decant the wash solution and add HRP-conjugated secondary antibody, diluted in wash buffer. Incubate for 30 minutes at 37° C, 1 hour at room temperature, or overnight at 4° C. Decant the antibody conjugate and wash for 40 minutes with agitation in wash buffer, changing the wash buffer every 10 minutes.

Substrate incubation (ECL)

Decant washing buffer and place the blot on a clean tray. Prepare enough detection substrate according to the manufacturer's specifications. Gently drop the substrate solution over the protein side of the membrane and incubate at RT between 1-5 mins. Remove the blot from the tray and place it between two pieces of write-on transparency film. Smooth over the covered blot to remove air bubbles and excess substrate and place the blot in an X-ray cassette. In the dark room lay x-ray film down over blots for 1sec - 20mins. Remove and develop film in the X-ray film processor. *All work must be carried out in the dark or only with red light.*

Reseal the cassette and replace all films into the respective box before switching on the lights.

Data analysis

Place the developed film into the UVP under white light and use the Bioimaging software to read the density of each band observed on the film.

The values are then normalised against the density of actin (or any other house-keeping protein) in the corresponding samples to obtain the expression of the protein in a particular cell line.

The results of histone deacetylase assay are shown in Table 4.

Table 4

Compound	Histone acetylation activities		
	Histone-3	Histone-4	Histone-2A
1	Active	Active	Active
2	Active	Active	Active
12	Active	Active	Not tested
SAHA	Active	Active	Active

Note: The histone acetylation activities of Compound 1, 2, and 12 2 are similar to that of SAHA

Histone H3 acetylation assay – ELISA approach

Quantitation of the acetylation histone3 can be measured by using an ELISA approach whereas the degree of histone acetylation can be monitored by a Western Blot approach, where specific antibodies directed against the acetylated version of histone H3 are used.

Briefly, 1.5×10^6 Colo 205 colon cancer cells were plated into 10 cm dishes and grown overnight in RPMI medium. Thereafter, the cells were treated with increasing amounts of HDAC inhibitory compounds by adding them into the medium (0.1, 1, 5 and 10 μ M final concentration). After 24 hours of incubation, the cells were harvested and lysed. The concentration of protein (described below) from lysed cells was determined. Protein lysate was subsequently subjected for ELISA or Western Blot procedure as described in detail below.

An Enzyme Linked Immunosorbent Assay (ELISA) can be applied to detect and quantify the acetylated histone3 (ACh3) in the protein lysate obtained from the Colo205 colon cancer cell line treated with the HDAC inhibitors. The basic material used in ELISA (1) is an antibody, either polyclonal antibody or monoclonal antibody where, in this case, it is specific for the ACh3 and histone3 (H3).

An ELISA assay was developed to detect H3 and ACh3. Different combinations of antibodies (see Table 5) that could be used as primary antibody (capture antibody) or secondary antibody were investigated to determine suitable antibodies and optimize antibody concentrations and assay conditions.

The combination of mouse monoclonal antibody against H3 and rabbit polyclonal antibody against ACh3 (Lys9/14) produced the best binding to the antigens, either peptides or protein lysate from Colo205 colon cancer cell line treated with the HDAC inhibitors. No background was observed. The detection antibody

used in this ELISA was donkey anti rabbit. The amount of the Ach3 in the protein lysates obtained from Colo205 colon cancer cell line treated with HDAC inhibitors (Figure 2) can be quantified. The current procedure for ELISA is described below

1. Coat the ELISA plate with 100 ul of 4 ug/ml of mouse monoclonal antibody against H3 at 4°C overnight
2. Wash the ELISA plate (x 5) with 200 ul of PBS containing 0.05% Tween-20
3. Block the plate with 150 ul of the Superblock solution (Pierce) at 37°C, 1 h
4. Wash the ELISA plate (x 5) with 200 ul of PBS containing 0.05% Tween
5. Dilute Ach3 peptide (used as a positive control and standard) in the first well, A1, to 25 ug/ml and perform serial dilution (2 fold) until A11
6. Dilute H3 peptide (used as a negative control) as step 4 in B1 and perform serial dilution (2 fold) until B11
7. Transfer 50 ul of the protein lysate from Colo205 colon cancer cell line and Colo205 colon cancer cell line treated with the HDAC inhibitors
8. Incubate the plate at 37°C, 1 h
9. Wash the ELISA plate (x 5) with 200 ul of PBS containing 0.05% Tween
10. Block the plate with 150 ul of the Superblock solution at 37°C, 1 h
11. Wash the ELISA plate (x 5) with 200 ul of PBS containing 0.05% Tween
12. Transfer 100 ul of 0.5 ug/ml of rabbit polyclonal antibody against Ach3 (Lys9/14) to the plate, A1- H11
13. Incubate the plate at 37°C, 1 h
14. Wash the ELISA plate (x 5) with 200 ul of PBS containing 0.05% Tween-20
15. Transfer 100 ul of the detection antibody, donkey anti rabbit (1:5000) to the plate, A1-H11
16. Incubate the plate at 37°C, 30 min
17. Wash the ELISA plate (x 5) with 200 ul of PBS containing 0.05% Tween-20
18. Transfer 100 ul of the substrate solution, 1 step-ABTS, into the plate, A1 – H12
19. Incubate the plate at 37°C, 1 h
20. Add 100 ul of 1% SDS to stop the reaction
21. Read at OD405 using spectromax

Equipment and materials

Equipment:

1. SPECTRAMax PLUS, Molecular Devices
2. Incubator, ITS Science and Medical Pte Ltd
3. Aspirator and vacuum

Materials:

1. ELISA plate (Immulon 2HB plate, Flat; Biolaboratories Pte Ltd)
2. Superclock buffer (Pierce Pte Ltd)
3. Antibodies (Table 1)
4. Acetylated histone3 (lys9/14) peptide and histone3 peptide (Upstate Pte Ltd)
5. Samples, protein lysate from Colo205 colon cancer cell line and Colo205 colon cancer cell line treated with the HDAC inhibitors
6. PBS buffer (Sigma Pte Ltd)
7. Tween 20 (Sigma Pte Ltd)
8. BSA (Sigma Pte Ltd)
9. 1-Step ABST (Pierce Pte Ltd)

Data analysis:

The standard curve (Figure 1) was drawn and the concentration of AcH3 [(Lys9/14), ug/ml] in a sample was determined using the Softmax software in spectromax. The amount of AcH3 [(Lys9/14), Figure2] in a sample was calculated based on the following formula;

$$\text{pg of AcH3 (Lys9/14) / ug of total protein total} = \frac{(\text{ug of AcH3 (Lys9/14) in the assay}) * 10^6}{\text{ug of protein in the assay.}}$$

Table 1: Antibodies used in the cross-species reactivity test and the combination antibodies studies

Antibodies used as either primary or secondary antibody	Detection antibody conjugated with HRP (horse radish peroxidase)
Rabbit polyclonal antibody against AcH3 (Lys9/14; Upstate Pte Ltd),	Donkey anti rabbit (Pierce Pte Ltd)
Rabbit polyclonal antibody against AcH3 (Lys14; Upstate Pte Ltd),	Goat anti rabbit (Pierce Pte Ltd)
Rabbit polyclonal antibody against AcH3 (Lys9, Upstate Pte Ltd),	Goat anti mouse (Pierce Pte Ltd)
Goat polyclonal antibody against AcH3 (Lys9/14, Santa Cruz Pte Ltd),	Rabbit anti goat (Pierce Pte Ltd)
Goat polyclonal antibody against H3 (N-20, Santa Cruz Pte Ltd)	Mouse anti goat (Pierce Pte Ltd)
Mouse monoclonal antibody against H3 (Upstate Pte Ltd)	

Figure 1: The standard curve of Ach3 peptide used for determination of the Ach3 concentration in a sample

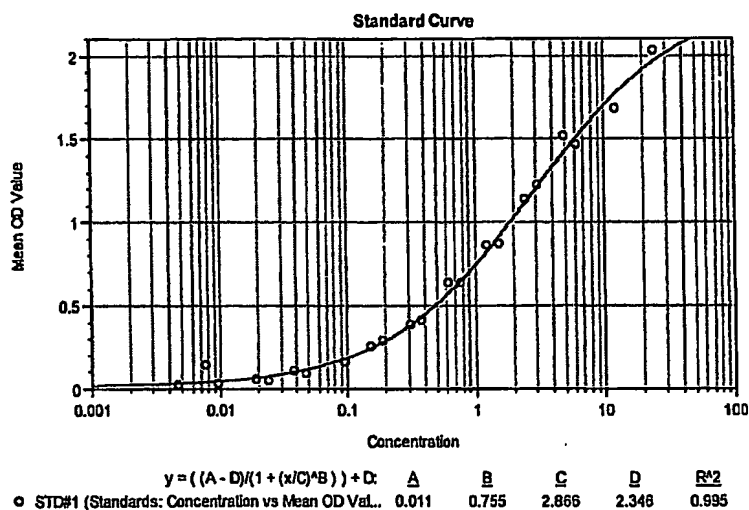
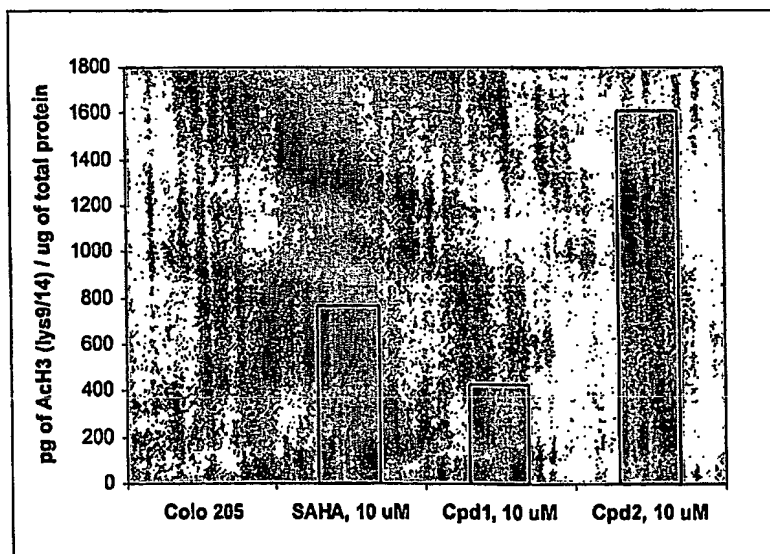


Figure 2: Quantitation of the Ach3 (lys9/14) from the protein lysate obtained from Colo205 colon cancer cell line treated with 10 μ M of different compounds



Reference:

Crowther JR (1995) ELISA theory and practice *In* Method in molecular biology vol. 42, Humana

Western Blot approach

Specific proteins can readily be identified with the use of antibodies directed explicitly towards it. Accordingly, the more abundant protein would display a stronger signal compared to one, which was present in a lower concentration.

Proteins must first be extracted from cells and quantified before equal amounts from each cell line can be separated by gel electrophoresis (SDS-PAGE).

Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for Western Blot analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane (nitrocellulose or PVDF) in the same sequence of separation as that on the SDS-PAGE. The membrane is then blocked with an inert protein like bovine serum albumin (BSA) or non-fat milk. This will avoid non-specific binding of the primary antibody to the un-blotted surface of the membrane.

To detect the antigen (separated protein of interest) blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane.

In order to detect the antibody, which has bound, an anti-immunoglobulin antibody coupled to a reporter group such as the enzyme horseradish peroxidase is added (e.g. Goat anti-human IgG- alkaline phosphatase). This anti-Ig-enzyme is commonly called a "second antibody" or "conjugate". Finally after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody is bound to the protein.

Protein extraction***Equipment:***

Centrifugation, Beckman Coulter Pte Ltd

Eppendorf centrifugation

Materials:

1. Cell Lysis buffer containing 150 mM NaCl, 1% Triton X-100, 50 mM Tris pH8, 1 mM EDTA and 1% protease inhibitor cocktail
2. Eppendorf tubes
3. 15 ml tubes
4. PBS buffer (Sigma Pte Ltd)
5. Versine (Gibco Pte Ltd)

Protein extraction from cells grown in culture:

1. Transfer the medium from the dish into 15 ml tubes and rinse cells in ice-cold PBS
2. Detach cells from the dish with 2 ml versine
3. Pellet cells by centrifugation ~1300 g, 5 min, and remove supernatant.
4. Resuspend cell pellet in 1 ml PBS buffer and transfer into a 1.5 ml Eppendorf tube and centrifuge ~13000 g for 10 min
5. Remove supernatant and gently resuspend cells in 500 ul of cell lysis buffer
6. Immediately freeze in LN₂ and allow to thaw at 37°C, 2 min.
7. Centrifuge at ~13000 g for 30 min
8. Transfer supernatant into a new eppendorf tube
9. Store the sample at -80° C or keep the sample on ice for further work.

Determination of protein concentration*Equipment:*

1. SPECTRAMax PLUS, Molecular Devices
2. Incubator, ITS Science and Medical Pte Ltd

Material:

1. Bicin solution (Sigma Pte Ltd)
2. Cu⁺⁺ solution (Sigma Pte Ltd)
3. BSA 1 mg/ml (Sigma Pte Ltd)
4. Protein lysate
5. PBS buffer
6. 96 well plate

Procedure:

1. Prepare protein standards of 1-500 ug/ml
2. Dilute samples, 1:5, 1:10 and 1:30, in PBS buffer
3. Prepare the reagent 1:50 (Cu:Bicin)
4. Transfer 200 ul reagent from step 3 into 96 well flat bottom plate
5. Transfer 20 ul of protein standard and diluted samples into the reagent
6. Incubate the plate at 37°C for 30 min
7. Let the plate cool down at room temperature, 5 min
8. Read the OD at 562 using Spectromax

Protein separation and transference*Equipment:*

Xcell Sure Lock Electrophoresis gel tank (In Vitrogen Pte Ltd)
BIORAD Mini Trans-Blot Electrophoretic Transfer Cell (BIORAD Pte Ltd)

Heat block

Materials:

1. 4 x sample loading buffer (In Vitrogen Pte Ltd)
2. 4-12% NuPage Bis-Tris gel (In Vitrogen Pte Ltd)
3. Blotting membrane 0.2um/0.45um (nitrocellulose or PVDF membrane)
4. Blotting Paper (thick) 7.5x10cm
5. NuPAGE Transfer Buffer
6. Methanol
7. MES SDS Running Buffer
8. SeaBlue marker (Invitrogen Pte Ltd)

Procedure:

1. Prepare all buffers solution and samples according to the Invitrogen NuPAGE Bis-Tris Gel Instruction Booklet
2. Set up the Xcell Sure Lock Electrophoresis gel tank according to the manufacturer's instructions
3. Place the pre-cast NuPage gels into the gel holders and add diluted NuPage tank buffer into the chambers
4. Load Seablue molecular weight marker and samples into respective wells in the gel
5. Connect the gel tank to the power pac and run the at 150 voltage constant for 1 hour
6. Prepare the PVDF membrane according to the manufacturing instruction
7. Take gel, dissemble and cut wells and stacking gel off
8. Make a gel sandwich (according to the BIORAD instruction) in the cassette
9. Transfer cassettes with the black side facing black part of blotting chamber
10. Fill chamber with transfer buffer past all holes of sandwich pack
11. Run at 100 voltage constant for 120 min at 4°C

Immunodetection (Antibodies)

Materials:

1. 10X Tris Saline Solution (TBS) (pH 7.6)

Tris 100 mM	12.1 g/L
NaCl 1.5M	87.6 g/L
Adjust pH to 7.6 and store at 4°C	
2. Blocking Solution (1x TSS/5% milk/1% BSA/0.1% Tween-20)

10x TBS	10 ml
Non-fat powder milk	5 g
BSA	1 g
Tween-20	100 ul
Deionised water	90 ml

3. Washing Buffer (1x TBS/0.5% milk /0.1% Tween-20), TBST

10X TBS	100ml
Non-fat powder milk	2.5g
Tween-20	1ml
Deionised water	890ml

4. Anti-acetylated Histone H3 (Lys14), Upstate Pte Ltd
5. Anti-Actin, Sigma Pte Ltd
6. Detection substrate, Pico or Femto (Pierce Pte Ltd)

Procedure:

1. Remove membrane from transfer apparatus
2. Wash membrane in TBS (x3) for 5 min at room temperature
3. Block membrane in blocking solution to saturate nonspecific sites
4. Incubate at room temperature with shaking, for 1 hour
5. Add primary antibody, Ach3 (lys14; 0.5 ug/ml) to the blocking buffer
6. Incubate at 4°C overnight with shaking.
7. Wash membrane 5 times in TBST, for 30min-1hr at room temperature
8. Incubate membrane with HRP conjugate secondary antibody (1:10,000) in blocking buffer
9. Incubate for 1hr at RT with gentle agitation
10. Wash membrane five times with TBST, for 5min each at room temperature
11. Prepare detection substrate (Peroxide Solution and Luminol/Enhancer Solution 1:1)
12. Incubate blot with detection substrate for 5 min.(Keep membrane wet at all times)
13. Remove membrane from detection substrate
14. Place membrane in between plastic sheet protector and press out bubbles between blot and surface of the membrane protector
15. Expose to film [exposure timing varies. General guidelines: $\geq 10\text{sec}$ (Femto) and $\geq 5\text{ mins}$ (pico)]

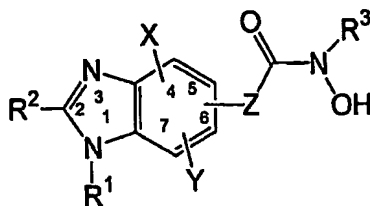
Data analysis:

1. Place the developed film into the UVP under white light and use the Bioimaging software to read the density of each band observed on the film.
2. The values are then normalised against the density of actin (or any other house-keeping protein) in the corresponding samples to obtain the expression of the protein in a particular cell line.

The details of specific embodiments described in this invention are not to be construed as limitations. Various equivalents and modifications may be made without departing from the essence and scope of this invention, and it is understood that such equivalent embodiments are part of this invention.

What is claimed is:

1. A compound of the formula (I)

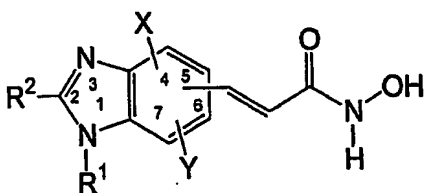


Formula I

wherein

- R¹ is selected from the group consisting of hydrido, C₁-C₁₀ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C₄-C₉ heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR⁴, -C(O)OH, -SH, and acyl;
- R² is H, halo, or is selected from the group consisting of C₁-C₁₀ alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C₄-C₉ heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; =O; =S; -CN; and -NO₂; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, -C(O)OR⁴, -C(O)OH, -SH, and acyl;
- R³ is selected from the group consisting of H, C₁-C₆ alkyl, acyl;
- X and Y are the same or different and are independently selected from the group consisting of hydrido, halo, C₁-C₄ alkyl, such as -CH₃ and -CF₃, -NO₂, -C(O)R⁴, -OR⁵, -SR⁵, -CN, and -NR⁶R⁷; X and Y are each attached to ring position 4, 5, 6 or 7 of Formula I
- R⁴ is selected from the group consisting of C₁-C₄ alkyl;
- R⁵ is selected from the group consisting of C₁-C₄ alkyl, heteroalkyl, acyl;
- R⁶ and R⁷ are the same or different and are independently selected from the group consisting of hydrido, C₁-C₆ alkyl, C₄-C₉ cycloalkyl, C₄-C₉ heterocycloalkyl, aryl, heteroaryl, arylalkyl, heteroarylalkyl;

- Z is a single bond or is selected from the group consisting of $-\text{CH}_2-$, $-\text{CH}_2\text{CH}_2-$, $-\text{CH}=\text{CH}-$, unsubstituted or substituted with one or more substituents independently selected from the group consisting of C_1 - C_4 alkyl; Z is attached to ring position 4, 5, 6, or 7 of Formula I; or a pharmaceutically acceptable salt thereof.
- 2. A compound of claim 1 wherein Z is a bond, $-\text{CH}_2-$, $-\text{CH}_2\text{CH}_2-$, or $-\text{CH}=\text{CH}-$, and Z is attached at ring position 5 or 6.
- 3. A compound of claim 1 wherein Z is $-\text{CH}=\text{CH}-$, and is preferably attached at ring position 5 or 6.
- 4. A compound of claim 1 wherein $\text{R}^3 = \text{H}$.
- 5. A compound of claim 1 wherein X and Y are hydrido groups.
- 6. A compound of claim 1 having formula Ib:



Formula Ib

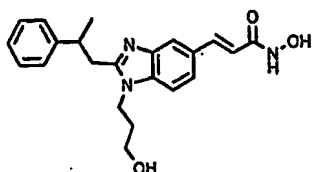
wherein

- R^1 is selected from the group consisting of C_1 - C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 - C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; $=\text{O}$; $=\text{S}$; $-\text{CN}$; and $-\text{NO}_2$; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, $-\text{C}(\text{O})\text{OR}^4$, $-\text{C}(\text{O})\text{OH}$, $-\text{SH}$, and acyl;
- R^2 is selected from the group consisting of C_1 - C_{10} alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, C_4 - C_9 heterocycloalkylalkyl, cycloalkylalkyl (e.g. cyclopropylmethyl), arylalkyl (e.g. benzyl), heteroarylalkyl (e.g. pyridylmethyl), unsubstituted or substituted with one or more substituents independently selected from the group consisting of: halogen; $=\text{O}$; $=\text{S}$; $-\text{CN}$; and $-\text{NO}_2$; and alkyl, alkenyl, heteroalkyl, haloalkyl, alkynyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, hydroxyl, hydroxyalkyl, alkoxy, alkylamino, aminoalkyl, acylamino, phenoxy, alkoxyalkyl, benzyloxy, alkylsulfonyl, arylsulfonyl, aminosulfonyl, $-\text{C}(\text{O})\text{OR}^4$, $-\text{C}(\text{O})\text{OH}$, SH , and acyl;

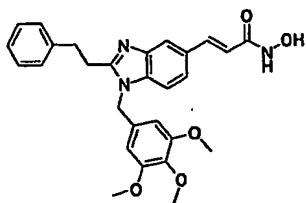
- X and Y are the same or different and independently selected from the group consisting of hydrido, halo, C₁-C₄ alkyl, such as -CH₃ and -CF₃, -NO₂, -C(O)R⁴, -OR⁵, -SR⁵, -CN, and -NR⁶R⁷; X and Y are each attached to ring position 4, 5, 6 or 7 of Formula 1b
- R⁴ is selected from the group consisting of C₁-C₄ alkyl;
- R⁵ is selected from the group consisting of C₁-C₄ alkyl, heteroalkyl, acyl;
- R⁶ and R⁷ are the same or different and independently selected from the group consisting of hydrido, C₁-C₆ alkyl, C₄-C₉ cycloalkyl, C₄-C₉ heterocycloalkyl, aryl, heteroaryl, arylalkyl, and heteroarylalkyl;

or a pharmaceutically acceptable salt thereof.

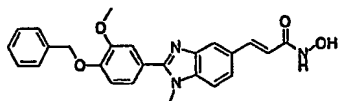
7. The use of a pharmaceutical composition comprising the compound of claim 1 to treat proliferative diseases, including cancerous tumors.
8. The use of a pharmaceutical composition comprising the compound of claim 6 to treat proliferative diseases, including cancerous tumors.
9. The use of a pharmaceutical composition comprising the compound of claim 1 to modify deacetylase activity, preferably histone deacetylase activity.
10. The use of a pharmaceutical composition comprising the compound of claim 6 to modify deacetylase activity, preferably histone deacetylase activity.
11. A pharmaceutical composition comprising the compound of claim 1 to modify activity, preferably HDAC1 activity.
12. A pharmaceutical composition comprising the compound of claim 6 to modify activity, preferably HDAC1 activity.
13. A pharmaceutical composition comprising the compound of claim 1 to modify activity, preferably HDAC8 activity.
14. A pharmaceutical composition comprising the compound of claim 6 to modify activity, preferably HDAC8 activity.
15. The compound of claim 6 wherein the structure of said compound is selected from compounds, and their pharmaceutically acceptable salts, selected from the group consisting of



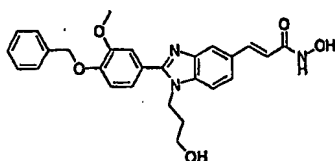
N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-(2-phenyl-propyl)-1H-benzimidazol-5-yl]-acrylamide



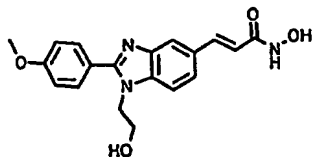
N-Hydroxy-3-[1-(3,4,5-trimethoxybenzyl)-2-(2-phenyl-ethyl)-1H-benzimidazol-5-yl]-acrylamide



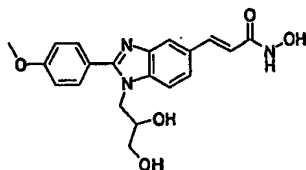
N-Hydroxy-3-[2-(4-benzyloxy-3-methoxy-phenyl)-1-methyl-1H-benzimidazole-5-yl]-acrylamide



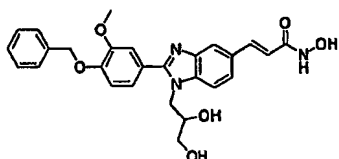
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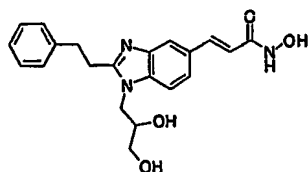
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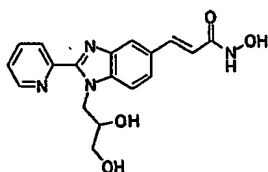
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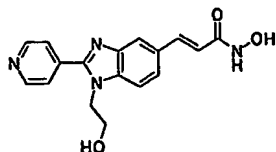
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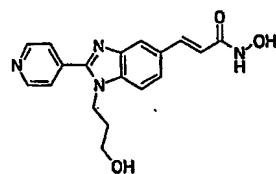
N-Hydroxy-3-[1-(2,3-hydroxy-propyl)-2-(2-phenyl-ethyl)-1H-benzimidazol-5-yl]-acrylamide



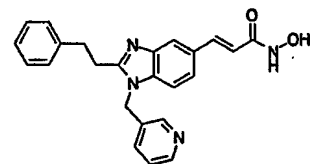
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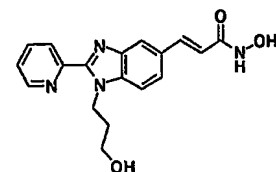
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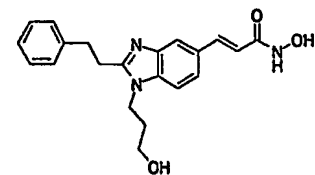
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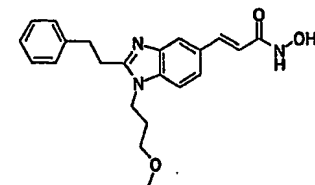
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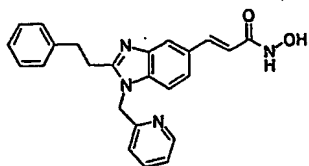
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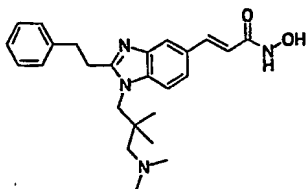
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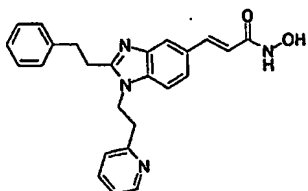
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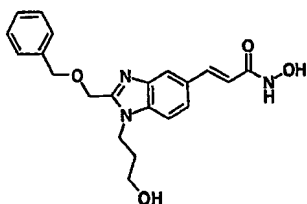
N-Hydroxy-3-(2-phenethyl-1-(pyridin-2-yl)methyl-1H-benzimidazol-5-yl)-acrylamide



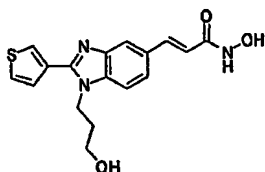
N-Hydroxy-3-[1-(3-Dimethylamino-2,2-dimethyl-propyl)-2-phenethyl-1H-benzimidazol-5-yl]-acrylamide



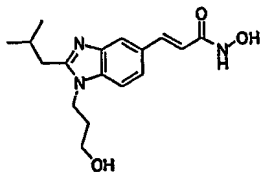
N-Hydroxy-3-[2-phenethyl-1-(2-pyridin-2-yl-ethyl)-1H-benzimidazol-5-yl]-acrylamide



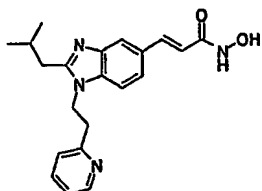
N-Hydroxy-3-[2-Benzyloxymethyl-1-(3-hydroxy-propyl)-1H-benzimidazol-5-yl]-acrylamide



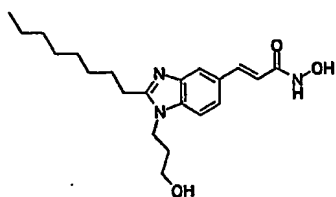
N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-thiophen-3-yl-1H-benzimidazol-5-yl]-acrylamide



N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-isobutyl-1H-benzimidazol-5-yl]-acrylamide

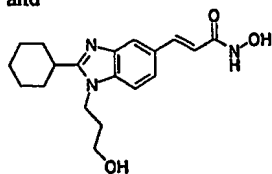


N-Hydroxy-3-[2-isobutyl-1-(2-pyridin-2-yl-ethyl)-1H-benzimidazol-5-yl]-acrylamide



N-Hydroxy-3-[1-(3-hydroxy-propyl)-2-octyl-1H-benzimidazol-5-yl]-acrylamide

and



N-Hydroxy-[2-cyclohexyl-1-(3-hydroxy-propyl)-1H-benzimidazol-5-yl]-acrylamide

16. An ELISA method to determine quantitation of acetylated histone, preferably histone 3, in extracts from eukaryotic cells.
17. An ELISA method to determine the potency of compounds that inhibit histone deacetylase in eukaryotic cells.

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